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A1

(54) Title: **PHARMACEUTICAL AEROSOL COMPOSITION**

(57) Abstract: An aerosol formulation comprising a biodegradable microsphere comprising a non-living reagent, such as a sub-unit vaccine, that produces a protective immune response in a mammal to whom it is administered. Nebulizers and inhalers containing such formulations are also described and claimed.

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PHARMACEUTICAL AEROSOL COMPOSITION

The present invention relates to pharmaceutical compositions, and in particular to compositions comprising immunogens, used in
5 the prophylactic and therapeutic treatment of infections.

The option to self administer vaccines by inhalation, for example using a nebulizer or inhaler such as a dry powder inhaler, would be advantageous from a logistical standpoint and
10 may be particularly effective for protecting individuals from pathogens that affect or utilise the respiratory tract as a portal of entry into the body.

US Patent No. 6,428,771 describes a method for controlled drug
15 delivery to the pulmonary system using microparticles incorporating the drug. Particles are described as having a diameter of from 0.5 and 10 μm . It is suggested that the drug may in fact comprise an antigen intended to elicit an a protective immune response. However this is not demonstrated.
20

Furthermore, administration of in particular non-living vaccines, such as sub-unit vaccines, has not yet been found to give effective protection using this mode of administration (see for example C.W. Purdy et al., Current Microbiology, (1998), 37,
25 p5).

The applicants have found that biodegradable microspheres, containing antigen, can engender immunological responses following delivery to experimental animals in the form of an
30 aerosol, provided the microspheres are of a type which are delivered most efficiently to the lung.

According to the present invention there is provided an aerosol formulation comprising a biodegradable microsphere of average
35 diameter of from 0.5 to 5 μm and comprising a non-living reagent

that produces a protective immune response in a host mammal to whom it is administered.

As used herein, the term "non-living reagent" refers to 5 immunogens such as polypeptides or proteins, which are derived for example from a pathogen such as a bacteria, virus or fungi. It also refers to inactivated microorganisms such as heat or chemically killed bacteria and/or viruses.

10 The term "aerosol" refers to a formulation that is deliverable in the form of a dispersion of a solid and/or liquid in a gas. These may be prepared from suspensions of the formulation in a liquid such as water, using a device such as a nebulizer, or from dry powders using a dry powder inhaler. In the case of the 15 nebulized aerosol, the dispersion comprises essentially wet microspheres in air.

The term "average diameter" as used herein, refers to the mean mass aerodynamic diameter of the microspheres. Mean mass 20 aerodynamic diameter is a measurement of particle size in an aerosol, which is the most relevant measurement when trying to predict if particles are respirable.

These formulations are effective in the administration of 25 reagents, which are capable of generating a protective immune response in an animal, particularly a mammal, to which it is administered. Examples of such agents include antigenic polypeptides as well as nucleic acid sequences which may encode these polypeptides and which are known as "DNA" vaccines.

30 Suitable polypeptides are sub-unit vaccines and others, such as diphteria toxoid, tetanus toxoid, *Botulinum* toxin FHC and *Bacillus anthracis* protective antigen (PA).

35 As used herein the expression "polypeptide" encompasses proteins or epitopic fragments thereof.

Suitable polypeptides are sub-unit vaccines.

- In a preferred embodiment, the formulation of the invention
5 comprises a biologically active agent which is capable of generating a protective immune response against *Yersinia pestis*. The agent is suitably a sub-unit vaccine, for example V antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these, or the F1 antigen of *Y. pestis* or an
10 immunologically active fragment thereof or a variant of these, or a combination of these. In particular as described in WO 96/28551, preferred vaccine comprises a combination of the F1 and V antigens.
- 15 As used herein, the term "fragment" refers to a portion of the basic sequence that includes at least one antigenic determinant. These may be deletion mutants. One or more epitopic region of the sequence may be joined together.
- 20 The expression "variant" refers to sequences of nucleic acids that differ from the base sequence from which they are derived in that one or more amino acids within the sequence are substituted for other amino acids. Amino acid substitutions may be regarded as "conservative" where an amino acid is
25 replaced with a different amino acid with broadly similar properties. Non-conservative substitutions are where amino acids are replaced with amino acids of a different type. Broadly speaking, fewer non-conservative substitutions will be possible without altering the biological activity of the
30 polypeptide. Suitably variants will be at least 60% identical, preferably at least 75% identical, and more preferably at least 90% identical to the base sequence. Identity in this case can be determined using available algorithms such as the widely used BLAST program.

The applicants have found that nebulization of PLA microspheres generates a respirable 'plume' of aerosolised particles, and this approach can be used to deliver immunogens to the respiratory tracts of experimental animals. Similar plumes 5 could be produced using other forms of inhaler such as dry powder inhalers.

Microspheres used are suitably small enough to allow them to be administered to the deep lung using a conventional nebulizer or 10 inhaler. For this purpose, microspheres will be less than 5 μm average diameter, preferably less than 3 μm average diameter, for instance from 0.5-3 μm , or more preferably from 1-3 μm and most preferably with an average diameter of between 1 and 1.5 μm .

15 Suitably 0% of microspheres have an aerodynamic diameter above 10 μm . More suitably, 0% of microspheres have an aerodynamic diameter above 9 μm , and preferably 0% of microspheres have an aerodynamic diameter above 6 μm .

20 Suitably, at least 90%, and preferably at least 95% of the microspheres in the formulation have an aerodynamic diameter of less than 5 μm , preferably with at least 80% of particles having a mean mass aerodynamic diameter of less than 3 μm .

25 By using microspheres of this size, efficient delivery of reagent into the deep lung is achieved. This is important in the delivery of reagents of this type as it is essential to achieve the highest concentrations of reagent, which can feasibly and safely be delivered in order to achieve the 30 protective immune response.

Microspheres are suitably biodegradable and are produced from 35 polymeric material. The polymeric material is suitably a biodegradable polymer other than a lipid, and in particular a biodegradable polyester. A particularly suitable polymer for

use in the preparation of microcapsules is Poly-lactide (PL) although other polymers such as poly(lactide-co-glycolide) PLGA may also be employed.

- 5 The microspheres may optionally further comprise agents which stabilise emulsions such as polyvinylalcohol (PVA), dipalmitoyl-phosphatidylcholine (DPPC), or methyl cellulose, and preferably polyvinylalcohol.
- 10 Suitably the non-living reagent is encapsulated within the microspheres (microcapsules). This again ensures the a high dose of the reagent is delivered to the lung which is important if a protective immune response is to be generated.
- 15 Microcapsules are suitably prepared using conventional methods such as the double emulsion/solvent evaporation method, as described for example by Beck et al., 1979, Fertility and Sterility, 31:545-551.
- 20 The encapsulation is suitably achieved using a double emulsion solvent evaporation method, in which a first emulsion is formed with the non-living reagent, and the structural polymer, mixing this with an aqueous phase (suitably without structural polymer) to form a secondary emulsion, evaporating solvent and isolating
- 25 small microspheres. In particular, the pharmaceutically active ingredient is dissolved or suspended in an aqueous solution, which optionally includes an emulsifier such as PVA. The emulsifier, where present is suitably included at low concentrations for example of less than 5%w/v. This solution or
- 30 suspension is then mixed with a solution of the high molecular weight structural polymer in an organic solvent such as dichloromethane. A primary emulsion is then formed, in particular by sonication of the mixture. The primary emulsion is then added to a secondary aqueous phase, which preferably
- 35 includes an emulsifier with vigorous stirring. Solvent is then preferably evaporated, conveniently at room temperature.

Microspheres can then be recovered, for example by centrifugation followed by lyophilisation.

The formulations of the invention may comprise microspheres per 5 se which are optionally preserved, for example by lyophilisation, or the microspheres may be combined with a pharmaceutically acceptable carrier or excipient. Examples of suitable carriers include solid carriers as is understood in the art for use in nebulizers.

10

In a particularly preferred embodiment, the formulation further comprises the non-living reagent in free form. The ratio of the amounts of the free reagent to the reagent associated with the microspheres used in the composition may vary depending upon the 15 particular agents being employed. Suitably the ratio of the free reagent to the reagent contained in the microspheres is in the range of from 1:20 to 2:1 and preferably at about 1:10.

20 The formulation of the invention may further comprise an adjuvant in order to enhance the immune response to the biologically active material administered. Suitable adjuvants include pharmaceutically acceptable adjuvants such as Freund's incomplete adjuvant, alhydrogel, aluminium compounds and, 25 preferably adjuvants which are known to up-regulate mucosal responses such as CTB, the non-toxic pentameric B subunit of cholera toxin (CT) or mutant heat-labile toxin (mLT) of *E.coli*. They may also include immunomodulators such as cytokines and CpG motifs.

30

Other adjuvant types are described in International Patent Application Nos. WO00/56282, WO00/56362 and WO00/56361.

Suitably the formulations are in unit dosage form. This will 35 vary depending upon the nature of the active agent being employed, the nature of the patient, the condition being treated

Further according to the invention there is provided a method of producing a protective immune response in a mammal in need thereof, said method comprising administering to the lung of 5 said mammal, a protective amount of an aerosol formulation as described above.

The invention will now be particularly described by way of example with reference to the accompanying diagrammatic drawings 10 in which:

- Figure 1 is a micrograph showing the morphology of microspheres prior to (A) and after (B) nebulization;
- 15 Figure 2 is a graph showing serum anti-V IgG endpoint titre in 6 BALB/c mice exposed to aerosolised microspheres containing recombinant *Yersinia pestis* V antigen; and
- 20 Figure 3 illustrates the survival of mice, previously exposed to aerosolised microspheres containing rV antigen, after subcutaneous injection of 6.5 MLDs *Y. pestis*;

25 Figure 4 is a fluorescence micrograph of lung taken 24 hours following exposure of mice to aerosolised microspheres loaded with FITC-BSA; and

Figure 5 is a fluorescence micrograph of lung lymph node taken 30 24 hours following exposure of mice to aerosolised microspheres loaded with FITC-BSA.

Example 1

Poly-lactide (resomer L210) microspheres containing either BSA or recombinant V antigen from *Y. pestis* were fabricated using a modified double-emulsion solvent evaporation process. PLA, sold 35 under the trade name Resomer L210, is a linear crystalline homopolymer with an inherent viscosity of approximately 3.6.

and other clinical factors. In general however, the formulations of the invention will comprise approximately 0.5 to 10 w/w of non-living reagent.

5 Exposed animals, in this case, mice, respond with a humoral response. It has also been found that experimental animals can be protected by this treatment from a lethal challenge with a pathogen such as the plague causing bacteria (*Yersinia pestis*) by exposure to aerosolised microspheres containing recombinant V
10 antigen. The applicants are therefore the first to demonstrate the successful aerogenic immunisation using non-living vaccines.

Dosages of the formulations of the invention will depend upon various factors such as the the nature of the patient, the
15 antigen used etc. and will be determined according to known clinical practice.

It has been found that in a particularly preferred embodiment, each administration of microsphere preparation to a mouse
20 contains from 1-100 μ g, suitably from 30-50 μ g and most preferably about 40 μ g of each of said antigens. Preferably the dosage to humans and mammals would be of the same order in terms of mg/Kg.

According to a further aspect of the invention, there is
25 provided a nebulizer or inhaler comprising a formulation as described above.

Dry powder inhalers may be particularly useful in the context of the invention as dry vaccine formulations, which would be used
30 therein, are stable at ambient temperatures.

In yet a further aspect, the invention provides the use of microspheres comprising a non-living reagent that produces a protective immune response in a mammal to whom it is
35 administered, in the preparation of a vaccine for administration as an aerosol.

The polymer was used at a concentration of 1.38%w/v in dichloromethane (10ml). An aqueous solution (0.5ml) containing the antigen of interest (about 4mg) was then added and the mixture stirred at high speed to generate an emulsion. This 5 emulsion was then added to a second aqueous phase and mixed together at high speed.

The solvent was then evaporated to leave an aqueous suspension of antigen-loaded microspheres.

10

Particles were aerosolised using a Sidestream® nebulizer. An aerosol particle sizer was used to analyse size characteristics. Samples were collected using a three stage liquid impinger and analysed using scanning electron microscopy, SDS PAGE and 15 western blotting procedures.

6 female BALB/c mice were exposed to a stream of aerosolised microspheres in a head only exposure line. 77mg of rV loaded microspheres were suspended in 17 ml of free V (at 0.4mg ml⁻¹ in 20 distilled water). Mice were exposed to the aerosolised microspheres for three ten minute runs, during which time approximately 3 ml of particle suspension was nebulized each run. The was repeated on days 0, 21 and 107 of the experiment and sera analysed for the presence of anti-V IgG using an 25 indirect ELISA. In order to assess the extent of protection afforded by inhalation of the V loaded microspheres, mice were injected subcutaneously with 6.3MLDs Y. pestis (GB strain) on day 136 of the experiment.

30 Results and Discussion

Microspheres had a loading of 3.8% w/w (BSA) and 3.3% w/w (rV). Following aerosolisation the BSA loaded particles had a mass median aerodynamic diameter of 1.3± 1.4µm, with 93% of the particles under 3µm. Following nebulization, particles retained 35 their morphology/topography (Figure 1) and contained antigenic material as detected by Western Blotting.

Although there was some inter-animal variation in the serum antibody response to aerosolised *Y. pestis* rV antigen, all 6 mice seroconverted after three immunising doses (Figure 2). Two 5 of the six mice responded with antibody titres that were of significant magnitude to confer protection from injected challenge with plague causing bacteria (Figure 3).

Example 2

10 Delivery of microencapsulated antigen to the lung and lung lymph node by aerosolisation

Poly-lactide (resomer L210, Alfa chemicals UK) microspheres, containing FITC-BSA were fabricated using a modified double-15 emulsion solvent evaporation process. Particles were aerosolised using a Sidestream® nebulizer (Profile, UK). Female BALB/c mice were exposed to the aerosolised microspheres in a head only exposure chamber. 24 hours following exposure mice were killed and their lungs and lung's lymph nodes were 20 extracted. Frozen sections were obtained from the extracted tissues using a cryostat. Frozen sections were examined for the presence of FITC-BSA loaded microspheres using a fluorescence microscope and the results for the lung and lung lymph nodes are shown in Figures 4 and 5 respectively.

25

The visualisation of punctate fluorescent material in the sections indicated the presence of FITC-BSA loaded microspheres in the lung and lymph nodes. These data support the tenet that microspheres can reach enter the lower respiratory tract 30 following nebulization. Furthermore, these data indicate that microspheres may be translocated from the lungs to the draining lymph nodes, following nebulization.

Claims

1. An aerosol formulation comprising a biodegradable microsphere of average diameter of from 0.5 to 5 μm comprising a non-living reagent that produces a protective immune response in a mammal to whom it is administered.
2. A formulation according to claim 1 wherein the said non-living reagent is antigenic polypeptide or a nucleic acid sequences which may encode such a polypeptide.
3. A formulation according to claim 2 wherein the said non-living reagent is a sub-unit vaccine.
4. A formulation according to any one of the preceding claims wherein the said non-living reagent is diphtheria toxoid, tetanus toxoid, *Botulinum* toxin Fc, *Bacillus anthracis* protective antigen (PA) or a polypeptide which is capable of generating a protective immune response against *Yersinia pestis*.
5. A formulation according to claim 4 wherein the non-living reagent is the V antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these, or the F1 antigen of *Y. pestis* or an immunologically active fragment thereof or a variant of these, or a combination of these.
6. A formulation according to any one of the preceding claims wherein the microspheres have an average diameter of less than 3 μm .
7. A formulation according to claim 6 wherein the microcapsules have an average diameter of between 1 and 1.5 μm .
8. A formulation according to any one of the preceding claims wherein the microsphere comprise a biodegradable polyester.

9. A formulation according to claim 8 wherein the polyester comprisesescomprise Poly-lactide (PL).

10. A formulation according to any one of the preceding claims 5 wherein the microcapsules are lyophilised.

11. A formulation according to any one of the preceding claims wherein the non-living reagent is encapsulated within the microspheres.

10

12. A formulation according to any one of the preceding claims which further comprises the non-living reagent in free form.

15

13. A formulation according to claim 12 wherein the ratio of the amounts of the free reagent to the reagent associated with the microspheres is in the range of from 1:20 to 2:1.

14. A formulation according to any one of the preceding claims in unit dosage form.

20

15. A nebulizer or inhaler comprising a formulation according to any one of the preceding claims.

25

16. The use of biodegradable microspheres having an average diameter of from 0.5 to 5 μ m and comprising a non-living reagent that produces a protective immune response in a mammal to whom it is administered, in the preparation of a vaccine for administration as an aerosol.

30

17. A method of producing a protective immune response in a mammal in need thereof, said method comprising administering to the lung of said mammal, a protective amount of an aerosol formulation according to any one of claims 1 to 14.

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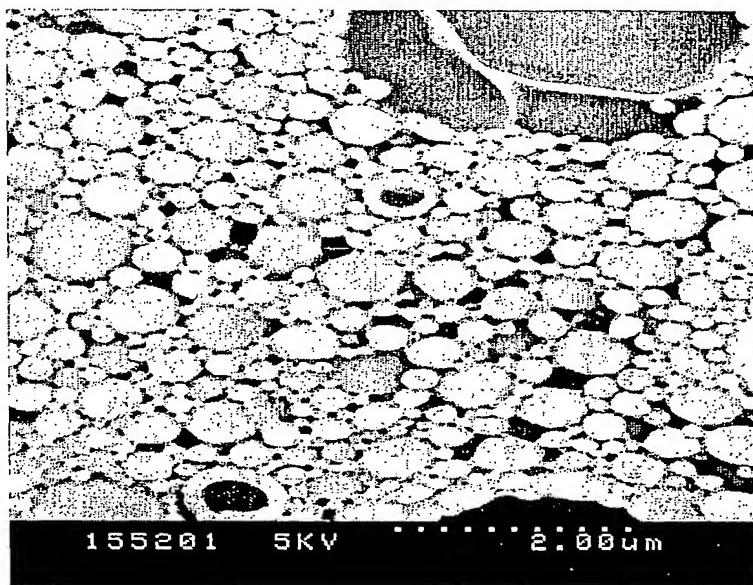
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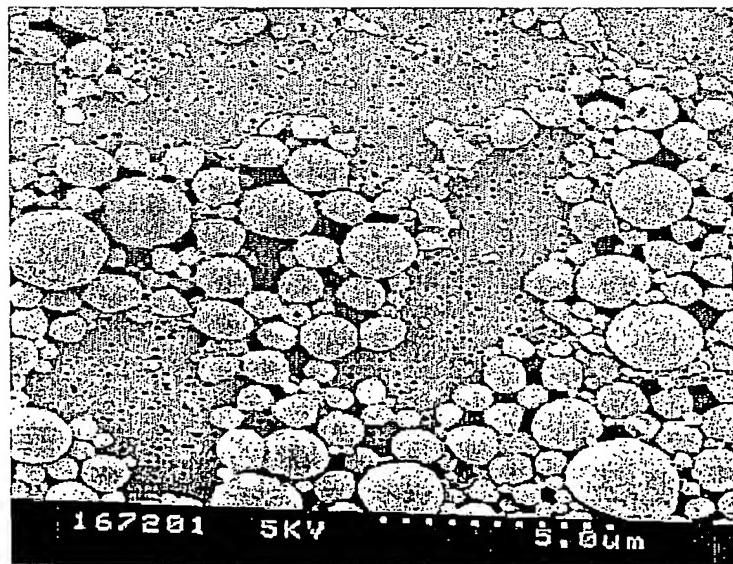
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Figure 1

A



B



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Figure 2

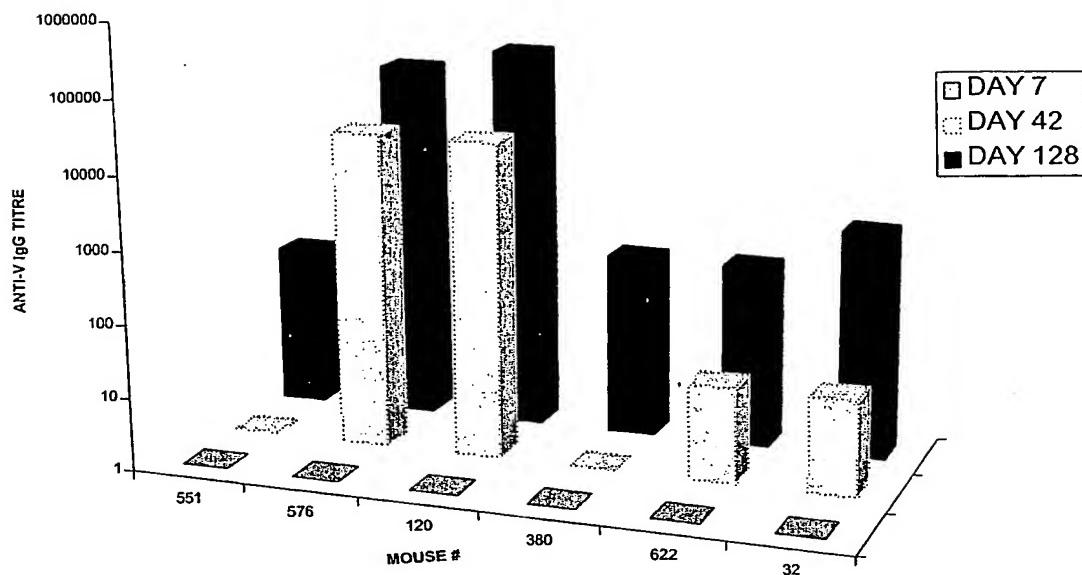
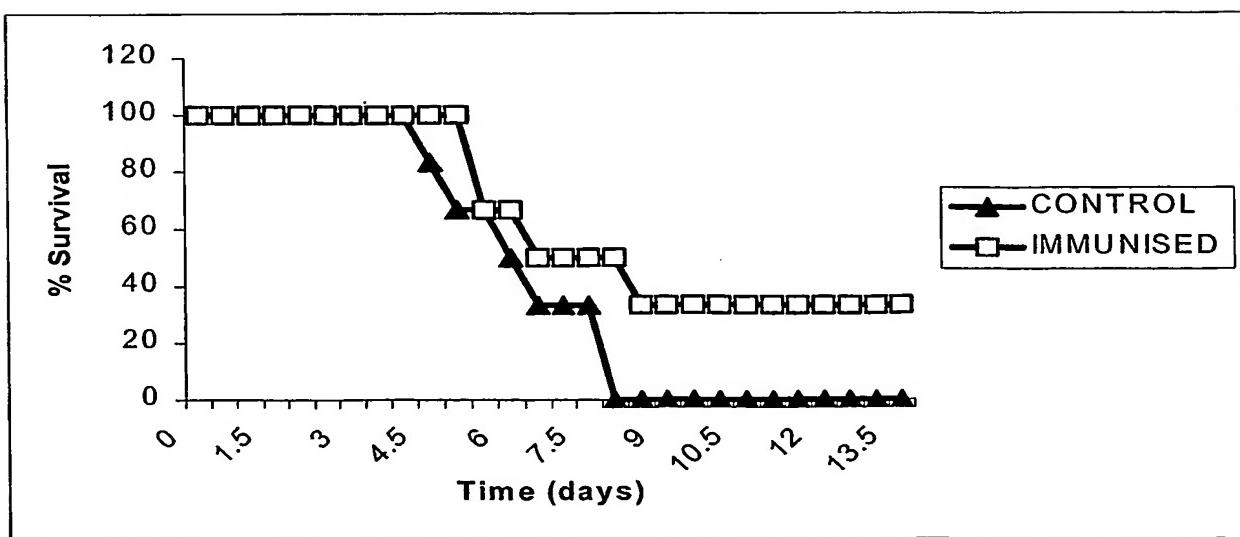


Figure 3



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Figure 4

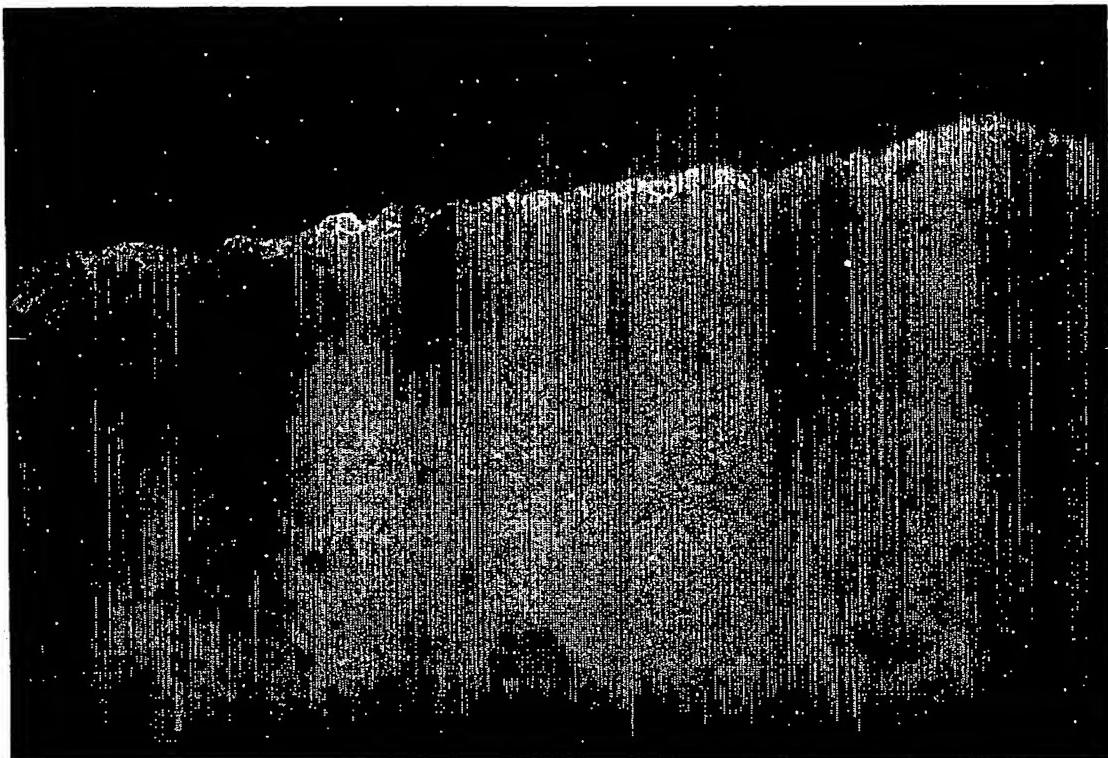
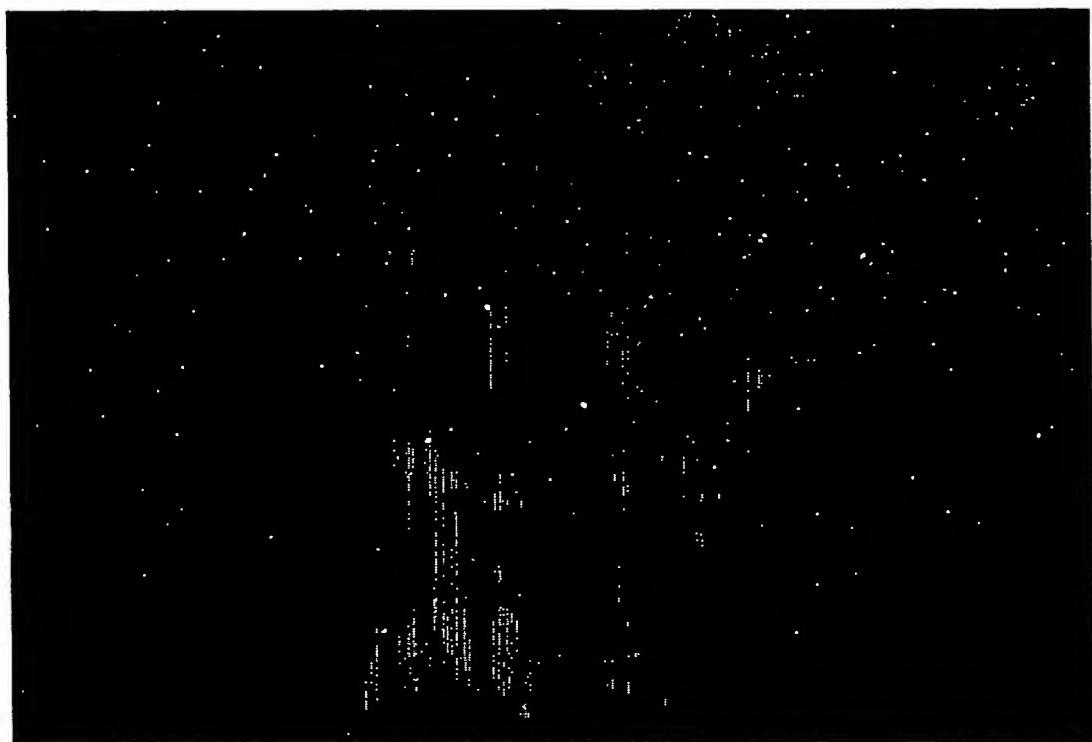


Figure 5



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INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/000104

A. CLASSIFICATION OF SUBJECT MATTER
 IPC 7 A61K9/72 A61K39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 02/43705 A (UNIV FLORIDA) 6 June 2002 (2002-06-06)</p> <p>page 3, line 35 – page 4, line 14 page 4, line 17 – line 39 page 5, line 11 – line 27 page 11, line 45 – page 12, line 38 page 13, line 8 – line 21 page 13, line 25 – page 14, line 6 examples claims</p> <p>-----</p> <p>-/-</p>	1,2, 6-11, 14-17

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>EYLES J E ET AL: "Protection studies following bronchopulmonary and intramuscular immunisation with Yersinia pestis F1 and V subunit vaccines coencapsulated in biodegradable microspheres: a comparison of efficacy" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 18, no. 28, August 2000 (2000-08), pages 3266-3271, XP004205869 ISSN: 0264-410X</p> <p>abstract</p> <p>paragraphs '02.2!', '02.3! paragraph '0004! -----</p>	1-11, 14-17
Y	<p>EYLES J E ET AL: "Analysis of local and systemic immunological responses after intra-tracheal, intra-nasal and intra-muscular administration of microsphere co-encapsulated Yersinia pestis sub-unit vaccines" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 16, no. 20, 1 December 1998 (1998-12-01), pages 2000-2009, XP004138449 ISSN: 0264-410X</p> <p>abstract</p> <p>page 2001, right-hand column, last paragraph - page 2002, left-hand column, paragraph 1</p> <p>page 2002, right-hand column, last paragraph</p> <p>page 2003, right-hand column, paragraph 5</p> <p>page 2008, right-hand column -----</p>	1-17
Y	<p>WO 01/70200 A (SECR DEFENCE ; ALPAR HAZIRE OYA (GB); EYLES JAMES EDWARD (GB); WILLIAM) 27 September 2001 (2001-09-27)</p> <p>page 4, line 14 - page 5, line 3</p> <p>examples</p> <p>claims -----</p>	1-17
X	<p>WILLIAMSON E D ET AL: "Local and systemic immune response to a microencapsulated sub-unit vaccine for plague" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 14, no. 17, 1 December 1996 (1996-12-01), pages 1613-1619, XP004016821</p> <p>ISSN: 0264-410X</p> <p>abstract</p> <p>page 1614, left-hand column, paragraph 4 - right-hand column, paragraph 4 -----</p>	1-11, 14-17

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INTERNATIONAL SEARCH REPORT

National Application No

PCT/GB2004/000104

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EYLES J E ET AL: "Microsphere translocation and immunopotentiation in systemic tissues following intranasal administration" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 19, no. 32, 14 September 2001 (2001-09-14), pages 4732-4742, XP004303168 ISSN: 0264-410X abstract paragraph '02.4! -----	1-11, 14-17

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Information on patent family members

National Application No

PCT/GB2004/000104

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